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FURTHER OBSERVATIONS ON VARIETIES OF STREPTOCOCCI WITH REFERENCE TO HEMOLYSIS

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The significance of whether strains of streptococci lake blood or not is of importance to the clinician from the standpoint of pathogenesis and to the bacteriologist from the standpoint of classification. The plate method has been the usual one used to determine whether or not strains of streptococci have hemolytic properties. Various types of hemolytic strains have been reported by different workers, especially by Smith and Brown.¹ They describe the alpha and beta types. Brown² has later reported another type of hemolytic zone on the blood-agar plate, the alpha prime type.

To standardize a method for general use the Medical Department of the United States Army³ in 1918 adopted the method of using a definite quantity of washed rabbit corpuscles with a definite quantity of a young rich broth culture of the strain to be tested. If hemolysis occurred the organism was considered as belonging to the hemolytic group. This method is more cumbersome, takes more work, and requires a longer time to determine whether or not a strain is hemolytic and should be justified only on superiority over the plate method or any other method requiring less time and energy.

In the work reported an effort has been made to study the ability of strains of streptococci grown on artificial medium to produce lysis of red blood cells and in doing this work a comparison of the army method and the plating method, when either rabbit or sheep blood is used, was made. The degree of hemolysis produced at the end of 2 hours by the method recommended by the United States Army is compared with the plate method, judging the hemolysis on the plate by the size and the clearness of the zone produced in from 18 to 24 hours.

¹ Jour. Med. Research, 1915, 31, p. 455.

² Monograph of Rockefeller Institute for Medical Research, No. 9, 1919.

³ Methods for the Isolation and Identification of *Streptococcus hemolyticus* Adopted by the Medical Department of the United States Army, New York, 1918.

Meat infusion agar with a reaction of 0.5% acid was used and to this 10% of defibrinated blood was added. One hundred and sixteen strains⁴ from various sources were tested. Of these 116 strains, 15 were from the blood of patients postmortem, 16 from tonsils removed in the dispensary, 51 from normal throats, 9 from normal horse feces, 7 from throats of influenza patients, 2 from pneumonia sputums, one from acute pharyngitis, one from pus from a frontal sinusitis, and 16 from cultures of throats which showed positive findings for diphtheria. These cultures, all except those isolated from diphtheria throats, were cultures that had been kept on artificial medium, meat infusion blood agar, for 3 years and transferred at intervals of 2 months. The work reported in this paper was done with strains picked from old cultures which had not been transferred for 4 months.

COMPARISON OF ARMY METHOD WITH PLATING METHOD WHEN RABBIT BLOOD WAS USED

Of the 15 cultures isolated from pathogenic processes, 14 gave a ++++ degree of hemolysis according to the army method. All of these 15 strains showed a zone of hemolysis on the rabbit blood-agar plate with a diameter of from 1.4 mm. The degree of clearness of hemolysis, with the exception of a few scattered colonies on the plates, ranged from 3 to 4 plus. Strain 114 gave no hemolysis by the army method but on plating on the blood agar a zone of hemolysis of 1 mm. in diameter and a +++ degree of clearness of hemolysis was produced. This strain was tested at other determinations with similar findings.

All of the 16 cultures isolated from removed tonsils gave a ++++ hemolysis according to the army method, except one. This one strain (52) gave a ++ degree of hemolysis. All of these 16 strains gave a 3 to 4 plus degree of clearness of hemolysis on the blood agar plate with zones ranging in diameter from 1 to 4 mm.

Of the 51 strains from the normal throats, 38 gave +++, 4 a ++, 2 a + degree of hemolysis by the army method, and 7 gave no hemolysis at all. On the blood agar plate all of these strains produced zones of hemolysis with a diameter of 0.5-3 mm. and a degree of clearness of 2 to 4 plus. All of the strains from the normal throat that gave no hemolysis by the army method produced zones of hemolysis on the rabbit blood-agar plate with diameters of 0.5 mm. and a high degree of clearness in the zone of hemolysis. Of the 38 strains giving a ++++ degree of hemolysis by the army method only 3 produced zones of hemolysis having an average diameter of less than 1 mm. On the other hand, the 7 failing to hemolyze by the army method, although producing small zones of hemolysis, produced a high degree of clearness in the small zone of hemolysis in the rabbit blood.

All of the 9 strains isolated from normal horse feces produced a ++++ degree of hemolysis according to the army method. All of these showed a zone of hemolysis on the blood agar plate with a diameter of 1-3 mm. and a 3 to 4 plus degree of clearness.

A ++++ degree of hemolysis was produced by the army method by all of the 11 strains from the throats of influenza patients (7), pneumonia sputum (2), acute pharyngitis (1), and a frontal sinusitis (1) with one exception, strain 413, which gave a + degree of hemolysis. All of these 11 strains

⁴ Jour. Infect. Dis., 1920, 26, p. 93.

hemolyzed blood on the blood-agar plate with a zone of hemolysis of 1-2 mm. in diameter. None of them dropped below a +++ degree of clearness of hemolysis in the hemolyzed zone.

All of the 16 strains recently isolated from diphtheria throats gave a ++++ degree of hemolysis by the army method with 2 exceptions. One (513) of these gave a ++ degree of hemolysis and one (501) failed to hemolyze at all. On the blood-agar plate all of the 16 strains produced zones of hemolysis varying from a 3 to 4 plus degree of clearness, with the exception of 2 in which ++ degree of clearness was produced.

COMPARISON OF SHEEP BLOOD IN BOTH ARMY AND PLATING METHODS

In the army method the use of rabbit blood is recommended. A comparison of sheep blood with rabbit blood was made with these 116 strains. When sheep blood was used with the 15 strains from necropsies, all but one strain produced a ++++ degree of hemolysis by the army method. This strain, 114, as with the rabbit blood, failed to produce any hemolysis at all by the army method. On the blood-agar plates the diameters of the zones of hemolysis as on the rabbit blood plate ranged from 1-4 mm. The number of large zones and the degree of clearness on sheep blood was slightly higher than on rabbit blood.

Of the 16 strains from tonsils only one (52) failed to produce a ++++ degree of hemolysis by the army method with sheep blood. The same results were obtained with rabbit blood. The size of the colonies on the blood-agar plate and the degree of clearness of the hemolyzed zones was slightly higher with the sheep blood.

Of the 51 throat strains 39 gave a ++++ hemolysis with the army method when using sheep blood as compared with 38 when using rabbit blood. Three gave a ++ with sheep blood as compared with 4 when rabbit blood was used. Seven failed to hemolyze sheep blood as compared with 7 that failed to hemolyze rabbit blood. As in strains isolated from other sources, these throat strains produced hemolysis in the blood-agar plate with a little higher average diameter and a greater degree of clearness in the hemolyzed zone when sheep blood was used.

As when rabbit blood was used, all the strains isolated from normal horse feces hemolyzed sheep blood to a ++++ degree by the army method. The size of the average diameter and the degree of clearness on the blood-agar plate with sheep blood was slightly higher than when rabbit was used.

Nine of the 11 strains from throats of influenza patients, pneumonia, sputums, etc., produced a ++++ degree by the army method with both rabbit and sheep blood. The size of the hemolyzed zones and the degree of clearness was as usual slightly greater with sheep blood.

All of the 16 strains isolated from diphtheria throats gave a ++++ hemolysis by the army method except one, as compared with the rabbit blood with which 3 failed to give a +++.

The comparison between rabbit and sheep blood when used with the army method may be summed up as follows: Of the 116 strains from various sources there were 10 failures to hemolyze by the army method when both rabbit and sheep blood were used, and the strains which failed to hemolyze were the same in both cases. This was

carefully checked. The 10 strains were grown in glucose blood broth for 2 weeks and retested with the same results. There was no complete failure to hemolyze on the blood-agar plate, although the size of the zones of hemolysis and the clearness of hemolysis in the zones varied. There is a slightly higher percentage of complete hemolysis with the army method when sheep blood is used than when rabbit blood is used in the ratio of 104 to 100.

PRODUCTION OF GREEN DISCOLORATION OF BLOOD BY HEMOLYTIC STREPTOCOCCI

Since Schotmüller's⁵ work with the blood-agar plate to differentiate strains of streptococci the question of hemolysis and the production of green discoloration on the blood-agar plate has been studied from various angles. Butterfield and Peabody⁶ found that pneumococci were able to convert oxyhemoglobin found in the red blood cells of the rabbit into methemoglobin. Cole⁷ states that the green produced about the colony by pneumococci on the blood-agar plate is methemoglobin. He also found that the pneumococci produced a hemotoxin seemingly of endotoxin nature⁸ and that this hemotoxin would lake red blood cells. Most observers of the green-producing streptococci and pneumococci on the blood-agar plate have noted that at 18-24 hours a green zone appears around the colony and that if observed later partial hemolysis can frequently be seen. There is a peripheral zone of green about the zone of hemolysis.

While plating out the series of 116 hemolytic strains of streptococci to determine whether they had retained their power to bring about hemolysis of the sheep blood, I noticed a few green colonies scattered among the typical beta hemolytic colonies on the sheep blood-agar plates. This led me to make observations on all the strains. Of the 116 strains observed, 54 showed from one to several green colonies scattered among the typical beta hemolytic colonies on the sheep blood-agar plates. The following table shows the number of strains in each group which produced some green colonies. It also shows the average size of the hemolytic zones on the blood-agar plates of those strains capable of producing green colonies as compared with the remaining strains which did not show the presence of green colonies scattered among the hemolyzers on the sheep blood-agar plates.

⁵ München. med. Wchnschr., 1903, 50, p. 849, 909.

⁶ Jour. Exper. Med., 1913, 17, p. 587.

⁷ Jour. Exper. Med., 1914, 20, p. 363.

⁸ Jour. Exper. Med., 1914, 20, p. 346.

RESULTS OF OBSERVATIONS ON ALL STRAINS

Source	Number of Strains	Strains with Green Colonies	Average Size of Hemolytic Zones of Strains Producing	
			Green Colonies Min.	No Green Colonies Min.
Necropsies.....	15	6	2.5	2.8
Removed tonsils.....	16	9	2.7	3.6
Normal throats.....	51	30	1.6	3.0
Influenza throats.....	7	2	2.0	2.7
Pneumonia, sputum, etc.....	4	2	2.0	2.0
Normal horse feces.....	9	0	...	3.5
Diphtheria throats.....	16	5	1.2	3.0

It is noted that strains from all sources gave green colonies except those strains isolated from normal horse feces. All of these strains were strong hemolyzers by the army method. More than 50% of the strains from tonsils and normal throats were capable of producing a few typical green colonies on the blood-agar plate. It was noticed throughout the work, as shown in the table, that there is a relation between the size of the clear zone of a strain and its ability to produce green colonies. The table shows an average diameter of the hemolyzed zone of those strains that produced green colonies to be 2 mm. in diameter as compared with 2.94 mm. of the average diameter of those strains that did not produce green colonies. It was also found that when a green colony was picked and replated that the tendency was to produce a smaller zone of hemolysis than was produced by the hemolyzers on the first plate.

To determine the nature and constancy of the green colonies produced, several strains were tested, with the following results:

147 A.

- Plate (a) plated from stock showed many hemolyzers and a few greens.
- Plate (b) plated from hemolyzer on plate (a) showed all hemolyzers.
- Plate (b') plated from green on plate (a) showed hemolyzers and 2 greens.
- Plate (c) plated from a green on plate (b') showed hemolyzers and few greens.
- Plate (d) plated from green on plate (c) showed hemolyzers and few greens.
- Plate (e) plated from green on plate (d) showed hemolyzers and few greens.
- Plate (f) plated from green on plate (e) showed hemolyzers.
- Plate (g) plated from small hemolyzer on plate (f) showed hemolyzers and a few slightly green colonies.
- Plate (h) plated from slightly green colony on plate (g) showed only hemolyzers.

Picked and replated small hemolyzers daily for 2 weeks. All colonies were hemolytic with one exception, an inactive colony. This colony remained inactive for 2 generations and then became hemolytic.

147 B.

- Plate (a) plated from stock showed all hemolyzers.
- Plate (b) plated from hemolyzer on plate (a) showed hemolyzers and greens.
- Plate (c) plated from hemolyzer on plate (b) showed hemolyzers and 2 green colonies.
- Plate (d) plated from a green on plate (b) showed only hemolyzers.
- Plate (e) plated from hemolyzer on plate (c) showed hemolyzers and 2 green colonies.
- Plate (f) plated from a green from plate (d) showed only hemolyzers.

508.

- Plate (a) plated from stock showed hemolyzers and greens.
- Plate (b) plated from a green on plate (a) showed hemolyzers and greens.
- Plate (c) plated from a green on plate (b) showed hemolyzers and one green.
- Plate (d) plated from green on plate (c) showed all hemolyzers.
- Plate (e) plated from hemolyzer on plate (d) showed all hemolyzers.

513 A.

- Plate (a) plated from stock showed hemolyzers and few greens.
- Plate (b) plated from a green on plate (a) showed hemolyzers and 2 greens.
- Plate (c) plated from a green on plate (b) showed all hemolyzers.
- Plate (d) plated from a hemolyzer on plate (c) showed all hemolyzers.

513 B.

- Plate (a) plated from stock showed hemolyzers and greens.
- Plate (b) plated from a green plate (a) showed hemolyzers and greens. Picked green from plate (b) and continued to replate daily for 2 weeks when inactive colonies appeared. These colonies when grown in dextrose broth for 24 hours and replated on blood agar showed only hemolyzers.

149 A.

- Plate (a) plated from stock showed all hemolyzers.
- Plate (b) plated from a good hemolyzer from plate (a) showed hemolyzers, greens and inactives.
- Plate (c) plated from a hemolyzer on plate (b) showed hemolyzers.
- Plate (d) plated from a green on plate (b) showed hemolyzers and greens.
- Plate (e) plated from an inactive on plate (b) showed inactives. One of these inactives was inoculated into dextrose broth for 2 transfers.
- Plate (f) plated from dextrose broth tube showed greens and hemolyzers.
- Plate (g) plated from a green on plate (f) showed small hemolyzers.
- Plate (h) plated from a hemolyzer on plate (g) showed small hemolyzers and one green.
- Plate (i) plated from the one green on plate (h) showed hemolyzers and one slightly green.
- Plate (j) plated slightly green from plate (i) and continued to replate daily for 2 weeks. At the end of this time all of the colonies were hemolytic.

The results suggest the possibility that hemolyzers may produce methemoglobin. Possibly the reason we do not detect the green is because it is overmasked by the hemolysis about the colony.

Holman⁹ reports that hemoglobin is in time changed to methemoglobin. Mann¹⁰ states that oxyhemoglobin is so readily converted into methemoglobin that if it be kept without special precautionary measures part of it becomes changed into methemoglobin. Webster¹¹ says that it is formed by the spontaneous decomposition of blood. Blake¹² states that the hemolytic streptococci cause complete hemolysis within 10-30 minutes but that in this time no methemoglobin is produced. Cole,⁷ in testing out various kinds of sugars to determine whether pneumococci cause the formation of methemoglobin only in the presence of glucose or that this sugar might be replaced by one with a different molecular configuration, found that methemoglobin was not produced by hemolytic streptococci. A method by which hemolysis could be stopped and at the same time the possibility of the production of methemoglobin not be interfered with was sought. Ruediger¹³ found that the addition of glucose to blood agar hindered or retarded the hemolytic power of hemolytic strains.

It has been found that on glucose blood-agar plates no hemolysis occurs but that colonies appear having from a typical green color to a reddish brown color about the colony. When blood is added to agar

⁹ Jour. Med. Research, 1916, 34, p. 377.

¹⁰ Chemistry of Proteids, 1906, p. 491.

¹¹ Diagnostic Methods, 1916, p. 411.

¹² Jour. Med. Research, 1917, 36, p. 99.

¹³ Jour. Infect. Dis., 1906, 3, p. 663.

containing no sodium chlorid, a transparent hemolyzed blood-agar plate results. Hemolyzers were grown on these plates. No signs of hemolysis could be noted and neither was there any visible indication by color on the blood-agar plate that methemoglobin was produced in 24 hours. After 48 hours a green color was noted about the colonies on the blood-agar plates. Blood was added to salt free plain broth. This gave a transparent hemolyzed fluid. This broth when inoculated with a hemolytic strain showed a greenish color in 24 hours. This blood broth culture was tested with the spectroscope after 24, 48, 72, and 96 hours of incubation at 37 C. and it was found that a typical methemoglobin spectrum was present. Hemolyzers were grown in salt blood broth and tested by the spectroscope and the typical methemoglobin band in the red of the spectrum was noted. In both of these conditions noninoculated controls were tested and gave no indications of the presence of methemoglobin either from gross appearance or by spectroscopic determination.

The method of hemolyzing blood by heat was tried. It was found that if 10% of defibrinated blood was added to salt agar at 80 C. and poured in a few seconds into a petri dish that the blood would show almost complete hemolysis. Such plates were inoculated with hemolyzers and incubated at 37 C. for 24 hours. Green-producing colonies appeared. Salt broth tubes, to which a similar amount of defibrinated blood was added when the broth was at 80 C. and immediately cooled, were inoculated with hemolyzers. After 24 hours a noticeable dark green color appeared in the medium and on spectroscopic examination the typical methemoglobin band in the red was present. Control uninoculated tubes were also tested with the spectroscope. These tubes showed the presence of methemoglobin. Blood was added to salt agar at boiling temperature and immediately plated. Fifty hemolyzers were stroked on the plates. In 24 hours typical green colonies were present. The intensity of the green increased when the plates were incubated for 48 hours. Nonhemolytic strains were also tested on this heated blood medium and produced colonies similar in all respects to those of the hemolyzers. Blood added to boiling salt broth was quickly cooled as in plating the agar. This blood broth was inoculated with hemolytic strains of streptococci and tested at 24, 48, and 72 hours of incubation at 37 C. The typical methemoglobin spectrum was found. Nonhemolytic streptococci were grown under similar conditions and tested with the same results. A noninoculated heated

blood broth tube was tested a few minutes after heating and no methemoglobin was detected. After incubating such a tube for 24 hours, methemoglobin could sometimes be detected.

Methemoglobin, according to the spectroscopic determination, appeared sometimes in uninoculated tubes of blood heated to either 80 C. or to boiling. Blood which had been added to sterile broth and had stood at room temperature for several weeks was tested with the spectroscope and the presence of methemoglobin was easily detected. It seems evident that the hemoglobin in the heated or unheated blood may on standing become changed to methemoglobin without bacterial action; but, considering the green on the heated or unheated blood-agar plate as an evidence of the presence of methemoglobin and the fact that unheated uninoculated red blood cells, hemolyzed or unhemolyzed, did not show indications of methemoglobin after 24 hours of incubation at 37 C. by the spectroscopic determination and that similar tubes inoculated with hemolytic streptococci did, it would seem that hemolytic strains may produce or may hasten the production of methemoglobin in the sheep red blood cells.

Cole⁷ decided that the best explanation of the bacterial process of producing methemoglobin from oxyhemoglobin is that there is at first a reduction process and then an oxidation process. It has been found (Mathews) that by heating oxyhemoglobin slightly the oxyhemoglobin is reduced gradually more nearly to hemoglobin (reduced hemoglobin). It is possible that by heating the blood at boiling for a few seconds some oxyhemoglobin is reduced to reduced hemoglobin and then hemolytic strains take up the process and oxidize the reduced hemoglobin to methemoglobin. Part of the hemoglobin may also be decomposed to globin and hematin. Mathews states that hemoglobin begins to decompose into hematin and globin at 64 C. It was found that not all of the hemoglobin was decomposed when heated to 80 C. or to boiling for a few seconds for in both cases the hemoglobin spectrum could be detected by spectroscopic determination. Globin is rich in various amino acids and probably for this reason produces a medium which is suitable for the good growth of streptococci. This medium has been found to be a good medium on which to grow Pfeiffer's bacillus.

The possibility that this green appearing on the heated blood-agar plate is sulphur-methemoglobin was considered. Sulphur-methemoglobin is produced by the union of hydrogen sulphid with hemoglobin

It is noticeable over the abdomen of persons brought to the necropsy table. This condition is known as pseudomelanosis. To a culture of hemolytic streptococci in blood broth in which typical methemoglobin bands were shown in the spectrum a few drops of dilute ammonium sulphid were added when the band immediately disappeared. This would indicate that the green produced by the hemolytic streptococci is methemoglobin and not sulphur-methemoglobin.

The fact that hemolytic streptococci may produce methemoglobin could account for strains described as the alpha type by Smith and Brown¹ in which the hemotoxin-producing power of the strain is weak and less hemolysis is produced and a slight amount of green is seen about the colony and the periphery of the hemolyzed zone. This fact could also explain Rosenow's observation of green colonies appearing among the hemolytic colonies and of my findings where strains which had been kept on artificial medium for more than 3 years were found which produced green colonies.

CONCLUSIONS

Hemolytic strains of streptococci when kept on suitable medium may retain the hemolytic property for at least 3 years.

The method recommended by the Medical Department of the United States Army does not seem to have any advantage over the plating method on the blood-agar plate in determining the degree of hemolysis. It seems possible to miss hemolytic strains by the army method when they can be detected by the plating method.

Sheep blood while it takes slightly more readily than rabbit blood can be used with equal efficiency in either the recommended army method or the plating method.

Typical hemolytic strains after being grown on artificial medium from 6 months to 3 years may produce colonies which show a green color about the colony similar in all appearances to the colonies of *Streptococcus viridans*.

The appearance of these green colonies tends to be associated with a weakening in the ability of strains to hemolyze as is shown by the degree of hemolysis produced by the army method and the smallness of the size of the hemolyzed zone about the colony on the blood-agar plate.

All of the 50 hemolyzers tested produced a green discoloration of the sheep red cells on heated blood similar in all respects to the green produced by nonhemolyzers.

This green colored substance seems to be methemoglobin when compared with the green produced on the blood-agar plate by *Streptococcus viridans* and according to the spectroscopic test.

Methemoglobin is more readily produced by hemolytic strains of streptococci in heated blood than in nonheated blood.